Bioorganic Chemistry 90 (2019) 103054

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Cytotoxicity of oleanolic and ursolic acid derivatives toward hepatocellular carcinoma and evaluation of NF-κB involvement

Gianfranco Fontana[a,*], Maurizio Bruno[a,*], Monica Notarbartolo[a], Manuela Labbozzetta[a], Paola Poma[a], Alberto Spinella[b], Sergio Rosselli[c]

[a] Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università degli Studi di Palermo, Viale delle Scienze, ed. 17, I-90128 Palermo, Italy
[b] Centro Grandi Apparecchiature (CGA) – ATeN Center, University of Palermo, via F. Marini 14, 90128 Palermo, Italy
[c] Dipartimento di Scienze Agrarie, Alimentari e Forestali (SAAF), Università degli Studi di Palermo, Viale delle Scienze, ed. 4, I-90128 Palermo, Italy

ARTICLE INFO

Dedicated to the memory of Dr. Simona Buccheri.

Keywords:
Ursolic acid
Oleanolic acid
HepG2
Hep3B
HA22T/VGH
Antitumor activity
NF − κB

ABSTRACT

Oleanolic and ursolic acids are two ubiquitous isomeric triterpene phytochemicals known for their anticancer activity. A set of derivatives of the two compounds with a modified oxidation state and lipophylicity at C-3 and C-28 positions, were prepared and tested as anticancer agents versus the lines HepG2, Hep3B and HA22T/VGH of hepatocarcinoma, a strongly aggressive tumor that is not responsive toward the standard therapies. New derivatives containing a three carbons side chain on the C-3 position were synthetized in both stereoisomeric forms by the Barbier-Grignard procedure and three of them were found to be active toward all of the three targets. The implication of the transcriptional nuclear factor NF − κB in the mechanism of action was assessed for the more active compounds in the set, as hepatocellular carcinoma (HCC) cyto-types are known to overexpress NF − κB.

1. Introduction

3β-hydroxy-olean-12-en-28-oic acid, known with the common name of oleanolic acid (OA, **1**) and 3β-hydroxy-urs-12-en-28-oic acid, known as ursolic acid (UA, **7**) are ubiquitous pentacyclic ursane- and oleanane-type triterpenes that possess the same functional groups, i.e. a carboxylic moiety at C-28, a β-hydroxy function at C-3 and a trisubstituted double bond between C-12 and C-13. The only difference between ursane and oleanane carbon framework lies in the position of 30-methyl (R_2 in OA **1** and R_3 in UA **7**, see Fig. 1). Both compounds were shown to possess several interesting biological properties, such as anti-inflammatory, antimicrobial, antidiabetic activities [1–4]. Ursolic acid is present in a large number of vegetables popularly known for their supposed tumor-preventive properties such as apples and cranberry [5] while OA **1** is normally assumed in daily diet through the consumption of other putative cancer-preventive foods, such as berry fruits [6] and olives (fruit and oil) [7]. Further evidences concern compounds **1** and **7** as components of the the active extracts of several medicinal plants with a recognized anti-cancer activity; recent examples include *Thymus mastichina* [8], *Hedyotis diffusa* [9], *Oldenlandia diffusa* [10] and *Cornus kousa* [11].

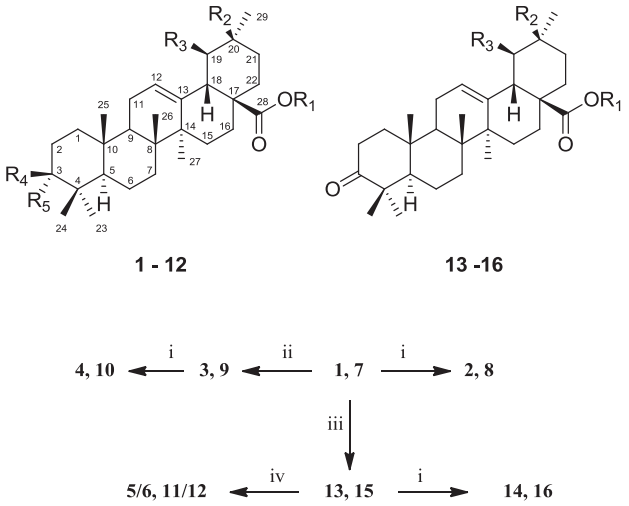
In recent years the in vitro and in vivo anti-cancer activity of the triterpene acids family, including compounds OA **1** and UA **7** and their semisynthetic derivatives, has been being investigated toward a great number of biological targets; the mechanisms of action are different and involve various stages of tumor development including apoptosis induction, cell cycle arrest, autophagy, invasion and metastasis inhibition, angiogenesis inhibition, etc. [12–14]. Further, OA **1** and UA **7** are well known for their hepatoprotective effects [15].

The nuclear factor-κB (NF-κB) is a transcription factor with multiple genetic targets that controls various cellular functions strongly overexpressed in hepatocellular carcinoma (HCC) [16,17]. It has been reported that UA and OA are able to inhibit NF-κB activation, and that this inhibitive effect is not cell type specific. HCC is a frequent and highly aggressive tumor, which in the advanced stages responds very poorly to currently available therapies [18,19].

The pathways involved after the inhibition of the NF-κB activation in the HCC model are not well understood and may include the inhibition of STAT3/6 and Akt/mTOR pathways [20]. Furthermore, UA apoptosis induction has been shown in Huh-7 cells with the

* Corresponding authors.
E-mail addresses: gianfranco.fontana@unipa.it (G. Fontana), maurizio.bruno@unipa.it (M. Bruno), monica.notarbartolo@unipa.it (M. Notarbartolo), manuela.labbozzetta@unipa.it (M. Labbozzetta), paola.poma@unipa.it (P. Poma), alberto.spinella@unipa.it (A. Spinella), sergio.rosselli@unipa.it (S. Rosselli).

https://doi.org/10.1016/j.bioorg.2019.103054
Received 1 March 2019; Received in revised form 29 May 2019; Accepted 6 June 2019
Available online 07 June 2019



Reagents and conditions: i. CH_2N_2, dry Et_2O, 0 °C, 1 h; ii. Ac_2O/Py 1:2, rt, 12 h; iii. Jones reagent, Me_2CO, rt, 30'; iv. Allyl-MgBr, dry THF, 0 °C to rt, overnight.

Compound	R_1	R_2	R_3	R_4	R_5
1	H	Me	H	OH	H
2	Me	Me	H	OH	H
3	H	Me	H	OAc	H
4	Me	Me	H	OAc	H
5	H	Me	H	OH	allyl
6	H	Me	H	allyl	OH
7	H	H	Me	OH	H
8	Me	H	Me	OH	H
9	H	H	Me	OAc	H
10	Me	H	Me	OAc	H
11	H	H	Me	OH	allyl
12	H	H	Me	allyl	OH
13	H	Me	H	-	-
14	Me	Me	H	-	-
15	H	H	Me	-	-
16	Me	H	Me	-	-

Fig. 1. Chemical structures and reaction conditions for the preparation of compounds **2–6**, **8–16** from **1** and **7**.

involvement of both the extrinsic and intrinsic pathways also inhibiting the p-Akt and p38 MAPK signaling transduction pathways [21]. As reported in the cited literature, even minimal modifications in the carbon framework, like the position of Me-30, as well as the oxidation state and the lipophilicity at C-3 of the triterpene acids, may result in a strong variation in the anti-cancer activity. Despite the great deal of data already available, a reliable prevision of the activity and the mechanism of action of a compound of this class toward a given target based on the already known structure-activity relations (SAR) is still far to be achieved. For this reason, it appears useful to test a set composed by derivatives of OA **1** and UA **7** that bear the same structural modifications applied on both the precursors in order to gain new SAR information about the cytotoxicity toward HepG2, Hep3B and HA22T/VGH cancer cell lines. In particular, we designed some chemical modifications at C-28 and C-3 changing the lipophilicity and/or the oxidation state at C-3. Further, a group of new derivatives of compounds **1** and **7**, that are compounds **5**, **6, 11** and **12**, bearing an allyl moiety at C-3 were synthetized and assessed for their bioactivity, in order to evaluate how an increase in the lipophilic character together with the maintenance of the hydrogen bonding ability of the OH group at C-3 can influence the cytotoxicity. It is worthy to note that some molecules in this set, i.e. compounds **3**, **9**, **13** and **15**, are natural occurring phytochemicals structurally related to compounds **1** and **7**; that were never evaluated for their activity toward Hep3B and HA22T/VGH cell lines. Further, none of the derivatives obtained from OA **1** and UA **7** were never evaluated for their NF-κB inhibition activity.

2. Results and discussion

OA **1** and UA **7** were obtained by Soxhlet extraction from *Olea europaea* leaves and *Malus domestica* fruit peel, respectively. Fig. 1 reports the chemical structures of compounds **1–16**, as well as the preparation of compounds **2–6**, **13** and **14** starting from **1** and **8 – 12**, **15** and **16** starting from **7.**

The diazomethane treatment of **1, 3, 7, 9, 13** and **15** gave quantitatively the methyl-esters **2**, **4, 8, 10, 14** and **16**. Both compounds **1** and **7** were converted to the corresponding acetates **3** and **9** by reaction with Ac_2O/Py at rt for 24 h. Oxidation of **1** by Jones method gave the keto-acid **13**, while its isomer **15** was obtained in the same experimental conditions from compound **7**.

The new C-3 epimers **5**, **6, 11** and **12** were obtained by the reaction of ketones **13** and **15** with 2.5 eqs. of allyl-magnesium chloride, followed by the usual work up. The chromatographic purification of the reaction mixtures led to isolate a more polar and more abundant

Table 1
^{1}H and ^{13}C NMR spectral data ($CDCl_3$) of compounds **5**, **6**, **11** and **12**.

	5		**6**		**11**		**12**	
H/C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C
1	1.44	36.64	1.35	34.28	1.46	36.85	1.41	34.50
	0.96		1.22		1.00		1.30	
2	1.65	28.37	1.67	28.69	1.66	28.43	1.70	28.78
	1.50		1.47		1.52		1.49	
3	–	76.25	–	75.10	–	76.22	–	75.06
4	–	41.10	–	40.47	–	41.11	–	40.47
5	1.02	53.08	1.34	50.92	1.01	53.09	1.34	50.94
6	1.48	18.87	1.47	18.73	1.47	18.84	1.47	18.73
	1.43		1.34		1.38		1.34	
7	1.44	32.78	1.48	32.57	1.48	33.06	1.54	32.91
	1.30		1.29		1.33		1.33	
8	–	39.29	–	39.21	–	39.51	–	39.42
9	1.60	48.08	1.68	47.50	1.55	47.98	1.63	47.44
10	–	37.11	–	36.98	–	37.08	–	36.90
11	1.89	23.38	1.89	23.22	1.91	23.28	1.93	23.10
	1.89		1.89		1.91		1.93	
12	5.28 t (3.4)	122.48	5.28 t (3.4)	122.66	5.24 t (3.3)	125.76	5.25 t (3.3)	125.90
13	–	143.66	–	143.58	–	137.92	–	137.88
14	–	41.53	–	41.59	–	41.83	–	41.95
15	1.73	27.69	1.71	27.62	1.88	27.99	1.89	27.94
	1.08		1.08		1.09		1.10	
16	1.98	22.90	1.98	22.87	2.01	24.03	2.01	24.06
	1.63		1.61		1.66		1.67	
17	–	46.54	–	46.52	–	47.93	–	47.96
18	2.83 dd (13.7, 4.1)	40.93	2.82 dd (13.7, 4.2)	40.87	2.19 d (11.1)	52.48	2.19 d (11.2)	52.54
19	1.63	45.92	1.61	45.84	1.35 m	39.01	1.34	39.01
	1.15		1.16					
20	–	30.65	–	30.65	1.02 m	38.81	1.00	38.80
21	1.34	33.80	1.36	33.77	1.52	30.58	1.52	30.60
	1.22		1.22		1.32		1.30	
22	1.77	32.42	1.77	32.44	1.71	36.70	1.72	36.72
	1.58		1.57		1.66		1.66	
23	0.90 s	19.45	0.81 s	20.66	0.90 S	19.52	0.81	20.72
24	0.87 s	24.10	0.93 s	23.58	0.89 s	24.14	0.94	23.59
25	0.96 s	15.81	0.91 s	14.88	0.98 s	15.95	0.93	15.06
26	0.76 s	17.17	0.75 s	17.20	0.78 s	17.13	0.78	17.14
27	1.16 s	26.01	1.15 s	26.08	1.10 s	23.71	1.10	23.74
28	–	184.05	–	184.06	–	183.78	–	183.46
29	0.91 s	33.06	0.91 s	33.05	0.88 d (6.0)	17.00	0.86 d (6.3)	16.99
30	0.94 s	23.57	0.93 s	23.58	0.96 d (6.1)	21.18	0.95 d (6.1)	21.17
1′	2.52 dd (14.1, 7.9)	37.63	2.41 dd (13.7, 7.5)	40.87	2.53 dd (14.1, 7.7)	37.60	2.41 dd (13.7, 7.5)	40.92
	2.23 dd (14.1, 6.7)		2.09 dd (13.7, 7.3)		2.23 dd (14.1, 6.8)		2.10 dd (13.7, 7.3)	
2′	5.91 dddd (17.2, 10.2, 7.9, 6.7)	134.91	5.92 dddd (17.0, 10.2, 7.5, 7.3)	134.64	5.92 dddd (17.3, 10.3, 7.7, 6.8)	134.91	5.93dddd (17.1, 10.2, 7.5, 7.3)	134.68
3′	5.15 brd (10.2)	118.04	5.18dd (10.2, 2.2)	118.94	5.16 brd (10.3)	118.14	5.18 dd (10.2, 2.0)	118.88
	5.12 brd (17.2)		5.11dd (17.0, 2.2)		5.14 br d (17.3)		5.11 br d (17.1)	

product (**5** from **13** and **11** from **15**), and a less polar, minor product (**6** from **13** and **12** from **15**). The structure assignments were made on the ground of spectroscopic analysis. For a matter of simplicity the following discussion will be focused on the determination of the structure and stereochemistry of oleanane-type compounds **5** and **6**. Closely similar considerations are applicable to ursane-type compounds **11** and **12**. In particular, the ^{1}H NMR spectra of both the compounds show similar signals pattern of the allyl moiety with a slightly difference of chemical shift of geminal vinyl protons that, in a 400 MHz ^{1}H spectrum, are partially overlapped in the compound **5** (δ 5.15 br d J = 10.2 Hz and 5.12 br d J = 16.0 Hz) while are separate signals (δ 5.18 dd J = 10.2, 2.2 Hz and 5.11 dd J = 17.0, 2.2 Hz) for the compound **6**.

The methine proton in the vinyl moiety has quite the same chemical shift being a multiplet at δ 5.92 and 5.91 for **5** and **6** isomers, respectively. A greater difference is observed for the allylic methylene showing two diastereotopic protons signals at δ 2.52 (dd, J = 14.1, 7.9 Hz) and δ 2.23 (dd, J = 14.1, 7.1 Hz) in the spectrum of compound **5** and at δ 2.41 (dd, J = 13.8, 7.4 Hz) and δ 2.09 (dd, J = 13.8, 7.4 Hz) in the spectrum of compound **6**. HSQC and HMBC experiments allowed to assign every signal of both compound **5** and **6** as reported in Table 1.

For the assignment of the correct C-3 configuration of these epimers, the literature data were initially considered. The comparison of the reported ^{13}C NMR spectra of OA [22] with 3-*epi*-oleanolic acid [23] showed that the signal of carbon C-3 of the β-OH epimer (oleanolic acid) had a downfield shift with respect to the same carbon signal of the 3-*epi*-oleanolic acid (α-OH).

The same trend can be reported for the signals of C-1 and C-4. On the base of this observation, the configuration of C-3 of the compound **6** can be assumed as α-OH therefore an *S* absolute configuration. On the converse, the compound **5** is the β-OH epimer having a *R* absolute configuration at C-3. The absolute configuration of C-3 was further supported by running a ROESy experiment on both compounds **5** and **6**. Two correlation cross peaks have been observed in the ROESy spectrum of the compound **6**. They involved the allyl protons with methyl groups in C-4. In particular, the proton at δ 2.41 correlated with Me at δ 0.93 whereas the proton at δ 2.09 correlated with the methyl signal at δ 0.81. For compound **5**, a clear correlation was observed between one of the allyl protons, specifically the proton resonating at δ 2.52, with H-5 (δ 1.02) having a biogenetic α configuration. Consequently, the correlations observed are compatible with structures showed in Fig. 2 in which the absolute configuration *R* at C-3 (β-OH) was assigned to the more polar and more abundant compound **5**, while the less polar minor

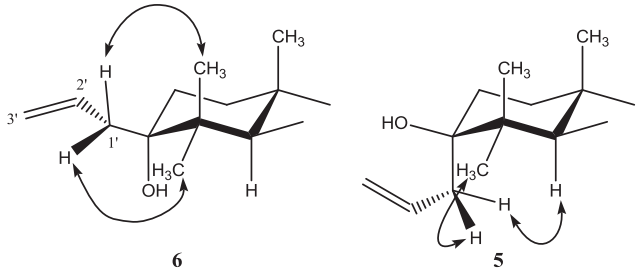


Fig. 2. Selected ROESy correlations for epimers **5** and **6**.

Table 2
IC_{50} values of the HCC cell lines treated with compounds **1–16**.

Compounds	HA22T/VGH	HepG2	Hep3B
1	> 100 μM	> 100 μM	> 100 μM
2	42.5 μM ± 0.7	40.0 μM ± 0.7	41.5 μM ± 2.1
3	> 100 μM	> 100 μM	> 100 μM
4	> 100 μM	> 100 μM	> 100 μM
5	31.0 μM ± 1.5*	28.0 μM ± 2.0*	32.5 μM ± 2.5*
6	35.8 μM ± 0.25*	44.0 μM ± 1.0*	33.7 μM ± 1.25*
7	23.1 μM ± 8.7	40.8 μM ± 11.9	52.0 μM ± 1.0
8	38.0 μM ± 4.0	45.0 μM ± 5.0	38.0 μM ± 2.0**
9	> 100 μM	> 100 μM	> 100 μM
10	> 100 μM	> 100 μM	> 100 μM
11	41.0 μM ± 12.7	45.0 μM ± 1.4	40.0 μM ± 2.8
12	> 100 μM	> 100 μM	> 100 μM
13	43.2 μM ± 5.25*	34.0 μM ± 1.5*	38.2 μM ± 1.75*
14	> 100 μM	> 100 μM	> 100 μM
15	> 100 μM	> 100 μM	90.7 μM ± 3.75*
16	> 100 μM	> 100 μM	> 100 μM

** $P < 0.05$.
* $P < 0.01$ *vs.* precursor compounds **1** and **7**.

product **6** was the other epimer *S* (α-OH).

The antitumor effects of all of the compounds **1–16** on HCC cell lines were evaluated by MTS assay. After 72 h of treatment the concentrations of the substance which caused 50% inhibition of cell growth are reported in Table 2. As it can be seen, the derivatives **2**, **5**, **6** and **13** are more active than their precursor **1** in all the cells tested. On the other hand, the derivatives of AU **7** show an activity similar to that of their precursor in both HA22T and HepG2 cells, while compounds **8** and **11** are more active in Hep3B cells.

Further, a comparison of the activities reported in table 2 highlights how the structural modifications imply comparable effects on the three cell lines, that may be both favorable or not: in particular, migration of Me-29, that means shifting from oleane to ursane series, has a significant positive effect when there is a OH function on C-3 (**7** *vs.* **1**), while the effect is negative when the C-3 is a carbonyl (**15** *vs.* **13**). On the other hand, methylation of the carboxylic moiety, as mentioned above, has a slight to negligible influence in the ursane set (compare **8** *vs.* **7**, **10** *vs.* **9** and **16** *vs.* **15**) while is moot in the oleane family, where the substitution of the carboxy function with a carbomethoxy one, may be favorable (**2** *vs.* **1**), unfavorable in the ketones (**14** *vs.* **13**), or irrelevant (**4** *vs.* **3**). Further, oxidation of C-3 implies a loss of activity in every case (**14** *vs.***2**, **15** *vs.* **7** and **16** *vs.* **8**) but for OA where this modification strongly enhances the cytotoxicity (**13** *vs.* **1**). Furthermore, the acetylation of the alcohol moiety at C-3 implies a drop in the cytotoxicity, in every case.

The kind of observed behavior is typical of a drug with a complex mechanism of action that concerns the involvement of multiple pathways and targets, as was already reported in literature for OA **1** and UA **7** (see references in "introduction" section).

The substitution of the C-3 proton in **1** and **7** with an allyl fragment is worth of further discussion. Four new molecules, that is two couples of epimers isolable in both the stereoisomeric forms were obtained: (*R*)-**5**, (*S*)-**6** from **1** and (*R*)-**11**, (*S*)-**12** from **7**.

In HepG2 cells, a slight difference in the cytotoxic effect observed for compounds **5** and **6** may be due to the stereochemistry at C-3; however, the stereochemistry at C-3 become relevant for compounds **11** and **12** in all of the cell lines tested.

We have evaluated the effects of the derivatives that have shown a cytotoxic activity in the three HCC cell lines (HA22TVGH, HepG2 and Hep3B), on the constitutive activation of NF-κB by TransAM assay after 24 h of treatment. As indicated in literature, our data have confirmed that OA **1** and UA **7** are inhibitors of NF-κB activation. The derivative of ursolic acid **8**, has shown inhibitory effects on NF-κB approximately comparable to those of UA **7** in all cell lines, while all the derivatives of OA **1** have strongly reduced NF-κB activation with respect to the precursor in all cell lines (Fig. 3).

It has been shown that the aggressiveness and the poor responsiveness of the HCC is due to the constitutive activation of NF-κB and its molecular targets such as MDR-1 (the multidrug resistance gene 1, which encodes for the P-glycoprotein), that was able to decrease intracellular drug concentration by acting as a drug efflux pump and the inhibitors of apoptosis proteins [16,17]. It is interesting to note that our three HCC cell lines are characterized by a high expression of IAPs and MDR-1 [24]. Little is known regarding the proapoptotic effects of OA **1** and UA **7** in HCC, however Wu et al. have shown that treatment with UA can sensitize HepG2 cells to cisplatin [25]. In HuH7 liver cancer cell, OA and UA induce apoptosis through a mitochondria-mediated pathway and downregulation of X-linked inhibitor of apoptotic protein (XIAP) also suppressed the activity of NF-κB [26].

3. Conclusions

A set of derivatives of OA **1** and UA **7** have been synthetized and tested toward three relevant HCC cell lines, HA22T/VGH, HepG2 and Hep3B getting some useful SAR information in vitro. This cancer is characterized by multidrug resistance, poor prognosis and by constitutively activation of NF-κB.

Four new derivatives with a side chain at C-3, i.e. epimeric compounds **5**, **6** from OA and **11**, **12** from UA, were obtained, the oleanane-type derivatives showing more activity than their precursor **1**.

We demonstrated for the first time that some derivatives of OA **1** and UA **7** inhibited cell growth and induced an inhibition of NF-κB activation in hepatocellular carcinoma cell lines. Interestingly, we observed a strong correlation between the cytotoxic activity and the NF-κB inhibition, in particular for compounds **5**, **6** and **13**.

Our findings, once again provide evidence that pharmacological inhibition of NF-κB may represent a promising approach for HCC therapy and further investigations are ongoing to clarify the molecular pathway downstream of NF-κB. Further investigations are in progress to clarify the involvement of NF-κB targets such as IAPs.

Furthermore, it can be hypothesized that pretreatment with this compounds could make cells more sensitive to the standard chemotherapeutic drugs and they could represent new possible therapeutic approaches for HCC.

4. Materials and methods

4.1. General

Reaction progress was monitored by TLC on silica gel plates (Merck 60, F_{254}, 0.2 mm). Organic solutions were dried over Na_2SO_4. Evaporation refers to the removal of solvent on a rotary evaporator under reduced pressure. ^{1}H-All samples were solubilized in deuterated chloroform ($CDCl_3$). The spectra were recorded at 300 K by a Bruker Avance II 400 spectrometer operating at 400.15 and 100.63 MHz for ^{1}H and ^{13}C, respectively. The instrument was equipped with an inverse broadband probe (BBI). For the acquisition of 1H spectra, a 11.87 μs 90° pulse, a delay time of 5 s and 16 scans were used. ^{13}C spectra were

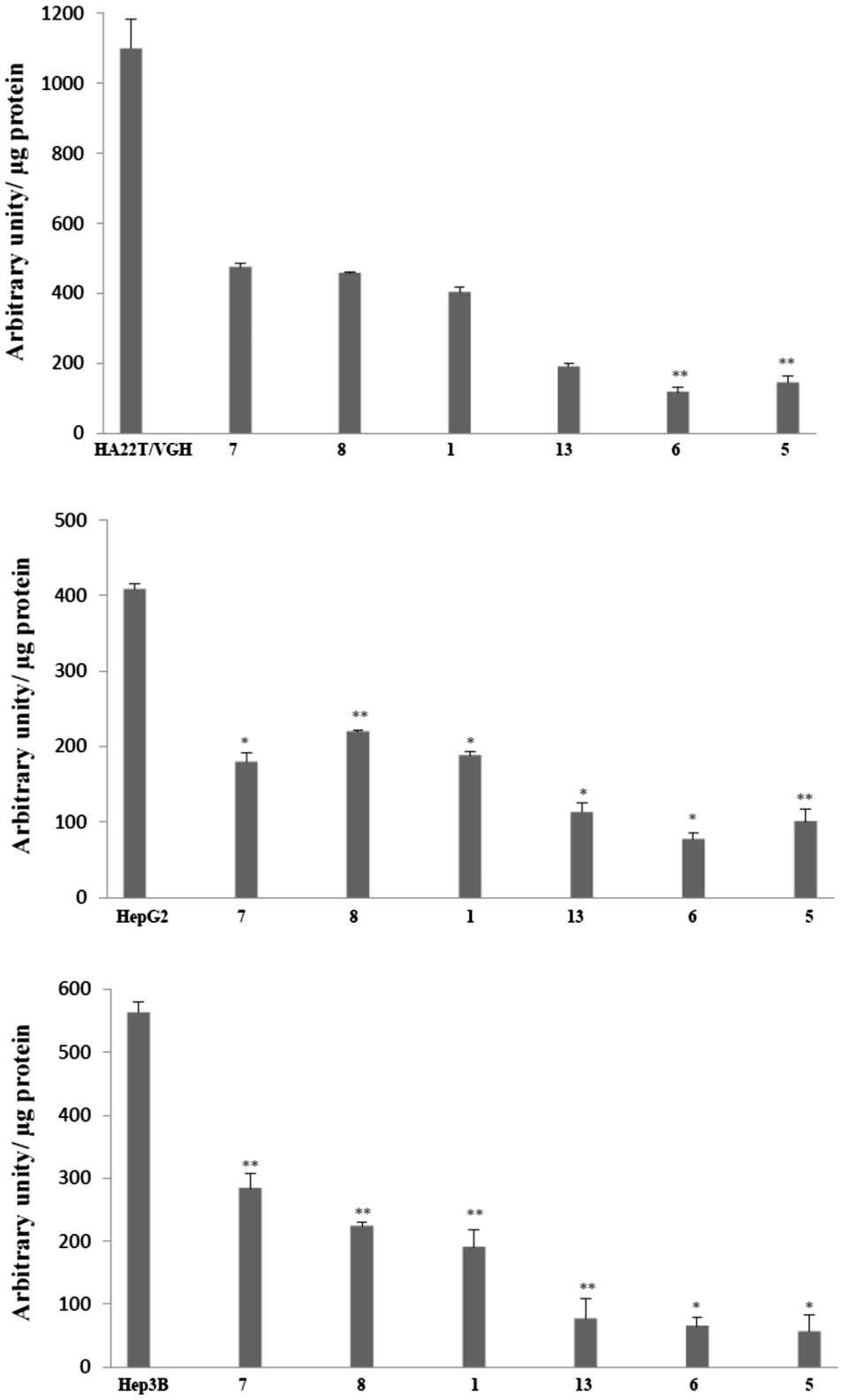


Fig. 3. NF-κB (p65 subunit) DNA binding capacity in nuclear extracts of HCC cell lines. The cells were treated for 24 h with compounds **1**, **5–8**, and **13**. Results are expressed as arbitrary units/µg protein of HA22T/VGH, HepG2 and Hep3B nuclear extracts and the mean ± SE of three independent observations. *P < 0.05 versus control; **P < 0.01versus control. For further details, see Materials and methods.

acquired using a 90° pulse of 12.2 µs, a decoupling pulse of 80 µs and a delay time of 3 s. The 2D ROESY spectra were acquired at 300 K using a spin-lock pulse of 300 ms, 8 scans and 256 experiments. The 2D HSQC correlation spectra were acquired at 300 K through a double inept transfer pulse sequence with decoupling during acquisition using a 90° pulse of 11.87 µs on the 1H nucleus, 8 scans and 256 experiments. The 2D HMBC long-range correlation spectra were acquired at 300 K through a J-filtered pulse sequence in order to suppress the single bond correlations with no decoupling during acquisition, using a 90° pulse of 11.87 µs on the 1H nucleus, 8 scans and 256 experiments. Merck silica

gel (Kiesegel 60/230–400 mesh) was used for flash chromatography (FC) columns. Optical rotations were measured by Jasco P-1010 digital polarimeter. Microanalysis data (C, H, N) obtained by an Elemental Vario EI. III apparatus were within ± 0.4% of the theoretical value. Dry THF was obtained by distilling commercial THF onto Na/benzophenone mixture.

Compounds **1–4, 7–10** and **13–16** were recognized by comparing physical and spectral properties with literature data.

4.2. Extraction and purification of compounds *1* and *7*.

1.0 Kg of *Olea europea* leaves collected in the campus garden were dried on air for one week, cut in little slices and charged into the Soxhlet apparatus and extracted with EtOAc for 6 h. Then the solvent was removed by rotavapor distillation and the extract was purified by column chromatography employing deactivated (with 15% V/W of H_2O) Merck silica gel (Kiesegel 60/70–230 mesh) as stationary phase and Me_2CO/*n*-hexane 2:8 as eluent. 1.30 g (0.13%) of pure OA **1** [22] were obtained.

2.0 kg of *Malus domestica* purchased in a local market were peeled and the peel was cut in little slices that were subsequently freeze-dried. The resulting material was subjected to the same isolation procedure described above obtaining 1.45 g (0.07%) of pure UA **7** [27].

4.3. General procedure for the methylation with CH_2N_2, preparation of compounds *2, 4, 8, 10, 14* and *16*.

To a solution of 0.5 mmol of the starting compound in 5 mL of THF, an ethereal solution of CH_2N_2 was added dropwise at 0 °C until a persistent pale yellow colour developed. The resulting solution was stirred for 30′. The CH_2N_2 excess was removed by gentle warming under hood and the solvent was evaporated. The residue was purified by FC ($CHCl_3$/MeOH 50:1) giving in quantitative yield reaction products identical in all respect to reported **2** [28], **4** [29], **8** [30], **10** [29], **14** [31] and **16** [32].

4.4. General procedure for the acetylation reaction; preparation of compounds *3* and *9*

300 mg of compounds **1** or **7** were solubilized in 3 mL of 1:2 Ac_2O/Py and stirred at rt for 24 h. Then the volatiles were removed by azeotropic evaporation with toluene. The residue was treated with 5 mL of 1:1 THF/H_2O and stirred for 3 h in order to hydrolyze *in situ* the mixed anhydride. The solvent was evaporated and the residue was purified by FC ($CHCl_3$/acetone 0.3% in acetone) giving 278 mg (85%) of a compound identical in all respect to reported **3** [33] and 9 [34].

4.5. General procedure for the Jones oxidations, preparation of compounds *13* and *15*

To a solution of 300 mg (0.64 mmol) of compound **1** (**7**) in Me_2CO, Jones reagent was added dropwise at 0 °C until a persistent pale yellow colour developed in the solution. The greenish solid residue was filtered off and the solvent was evaporated. The residue was purified by FC (light petroleum/acetone 5% in acetone) to give 195 mg (65%) of a compound identical in all respect to reported **15** [34], from **7** and 265 mg (89%) of a compound identical in all respect to reported **13** [35], from **1**.

4.6. Preparation of stereoisomers *5, 6* and *11, 12* by Grignard addition reaction on ketones *13* and *15*.

To a solution of 250 mg (0.55 mmol) of compound **13** (**15**) in 15 mL of dry THF, 700 mL (1.4 mmol) of 2 M allylmagnesium chloride in THF were slowly added via syringe under an Ar atmosphere at a temperature of 0 °C. The mixture was left to warm until rt overnight and then was poored into an iced 1 M HCl solution. The resulting mixture was stirred for 30′ while adjusting the pH to 2 in order to protonate the carboxylic function. Then the mixture was extracted with $CHCl_3$ (4 X 30 mL), the collected organic phase was neutralized with water and dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue was purified by FC (cyclohexane/acetone 9:1) giving 28 mg (10%) compound **6** and 96 mg (35%) of compound **5**.

*(3α)-allyl-(3β)-hydroxyolean-12-en-28-oic acid (**5**):* amorphous white solid, *anal.* C 79.11%, H 10.81%; calcd for $C_{33}H_{52}O_3$, C 79.79%, H 10.55%. $[\alpha]_D^{25}$ + 55.4 (*c* = 0.01, $CHCl_3$); ^{1}H NMR and ^{13}C NMR: see Table 1.

*(3β)-allyl-(3α)-hydroxyolean-12-en-28-oic acid (**6**):* amorphous white solid, *anal.* C 79.89%, H 10.77%; calcd for $C_{33}H_{52}O_3$, C 79.79%, H 10.55%. $[\alpha]_D^{25}$ + 31.4 (*c* = 0.0026, $CHCl_3$); ^{1}H NMR and ^{13}C NMR: see Table 1.

*(3α)-allyl-(3β)-hydroxyurs-12-en-28-oic acid (**11**):* amorphous white solid, *anal.* C 79.55%, H 10.80%; calcd for $C_{33}H_{52}O_3$, C 79.79%, H 10.55%. $[\alpha]_D^{25}$ + 29.0 (*c* = 0.136, $CHCl_3$); ^{1}H NMR and ^{13}C NMR: see Table 1.

*(3β)-allyl-(3α)-hydroxyurs-12-en-28-oic acid (**12**):* amorphous white solid, *anal.* C 79.80%, H 10.60%; calcd for $C_{33}H_{52}O_3$, C 79.79%, H 10.55%. $[\alpha]_D^{25}$ + 40.0 (*c* = 0.3, $CHCl_3$); ^{1}H NMR and ^{13}C NMR: see Table 1.

4.7. Cell cultures

The culture medium of human HCC cell lines is Roswell Park Memorial Institute (RPMI) 1640, supplemented with 10% heat-inactivated fetal calf serum, 2 mML-glutamine, 1 mM sodium pyruvate, 100U/mL penicillin, and 100 μg/mL streptomycin (all reagents were from HyClone Europe Ltd., Cramlington, U.K.); the cells are cultured in a humidified atmosphere at 37 °C in 5% CO_2 and routinely tested for Mycoplasma contamination. HA22T/VGH cell line was kindly provided by Professor M. Levrero (Laboratory of Gene Expression, Fondazione Andrea Cesalpino, University of Rome 'La Sapienza', Rome, Italy), Hep3B and HepG2 cell lines were obtained from the American Type Culture Collection (ATCC). After obtaining the cells, the first passage carried out was assigned passage number 1 and having a narrow range of passage numbers were used for all experiments.

4.8. Cell growth assays

For MTS assay we estimate cell growth inhibition as a percentage of the absorbance measured in the control cells. The cells were seeded onto 96-well plates at 2 × 104 cells/well and after 24 h the compounds were added in different concentrations. After 72 h, 15 μl of a commercial solution containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate (obtained from Promega Corporation, Madison, WI, USA) were added and after a period variable for each cell line, the bioreduction of the MTS dye was assessed by measuring the absorbance of each well at 490 nm.

4.9. NF-κB activation

NF-κB activation was evaluated by the DNA-binding capacity of NF-κB (p65 subunit) using the TransAM™ NF-κB and Nuclear Extract™ Kits (Active Motif, Carlsbad, CA, USA). The assay is based on the measure of binding between an oligonucleotide containing the NF-κB consensus binding site (5′-GGGACT TTCC-3′) and the activated NF-κB contained in the nuclear extracts of cells treated. In particular, the NF-κB bound to the oligonucleotide is detected using an antibody directed against an epitope on p65 that is accessible only when NF-κB is bound to its target DNA. Addition of a secondary antibody conjugated to horseradish peroxidase provides sensitive colorimetric readout that is quantified by

densitometry. The results were expressed as arbitrary units: one unit is the DNA binding capacity shown by 2.5 μg of whole cell extract from Jurkat cells stimulated with 12-O-Tetradecanoylphorbol-13-acetate (TPA) + calcium ionophore (CI)/μg protein of nuclear extracts. The specificity of the assay is defined by contemporaneous incubations in presence of an excess of the non-immobilized consensus oligonucleotide, as a competitor, or of a mutated consensus oligonucleotide.

4.10. Statistical analysis

Results are presented as mean ± standard deviation. The significance of differences between means was evaluated by Student's *t*-test. A statistically significant difference is indicated by $p < 0.01$ and $p < 0.05$.

Funding

This work was supported by grant from MIUR-ITALY PRIN2017 "STOLEN MOLECULES - Stealing natural products from the depot and reselling them as new drug candidates" (Project N. 2017A95NCJ).

Acknowledgements

We are grateful to MIUR PRIN for financial support. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data were provided by Centro Grandi Apparecchiature –UNINetLab - Università di Palermo funded by P.O.R. Sicilia 2000-2006, Misura 3.15 Quota Regionale. Project PON a3_00053-PLASS are gratefully acknowledged for the supply of the MS apparatus.

Declaration of Competing Interest

None.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103054.

References

[1] I.T. Babalola, F.O. Shode, Ubiquotous ursolic acid: a potential pentacyclic triterpene natural product, J. Pharmacogn. Phytochem. 2 (2013) 214–222.
[2] A. Paszel-Jaworska, A. Romaniuk, Maria Rybczynska, Molecular mechanisms of biological activity of oleanolic acid - a source of inspiration for a new drugs design, Mini-Rev. Org. Chem. 11 (2014) 330–342, https://doi.org/10.2174/1570193X1103140915111839.
[3] H. Chen, Y. Gao, A. Wang, X. Zhou, Y. Zheng, J. Zhou, Evolution in medicinal chemistry of ursolic acid derivatives as anticancer agents, Eur. J. Med. Chem. 92 (2015) 648–655, https://doi.org/10.1016/j.ejmech.2015.01.031.
[4] Y.Q. Meng, D. Liu, L.L. Cai, H. Chen, B. Cao, Y.Z. Wang, The synthesis of ursolic acid derivatives with cytotoxic activity and the investigation of their preliminary mechanism of action, Biorg. Med. Chem. 17 (2009) 848–854, https://doi.org/10.1016/j.bmc.2008.11.036.
[5] C.C. Neto, Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases, Mol. Nutr. Food Res. 51 (2007) 652–664, https://doi.org/10.1002/mnfr.200600279.
[6] I. Svarcova, J. Heinrich, K. Valentova, Berry fruits as a source of biologically active compounds: the case of Lonicera caerulea, Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech. Repub. 151 (2007) 163–174, https://doi.org/10.5507/bp.2007.031.
[7] C. Sanchez-Quesada, A. Lopez-Biedma, F. Warleta, M. Campos, G. Beltran, J.J. Gaforio, Bioactive properties of the main Triterpenes found in Olives, Virgin Olive Oil, and Leaves of *Olea europaea*, J. Agric. Food Chem. 61 (2013) 12173–12182, https://doi.org/10.1021/jf403154e.
[8] J. Gordo, P. Maximo, E. Cabrita, A. Lourenco, A. Oliva, J. Almeida, M. Filipe, P. Cruz, R. Barcia, M. Santos, H. Cruz, *Thymus mastichina*: chemical constituents and their anti-cancer activity, Nat. Prod. Commun. 7 (2012) 1491–1494 PMID: 23285814.
[9] H.Z. Lee, D.T. Bau, C.L. Kuo, R.Y. Tsai, Y.C. Chen, Y.H. Chang, Clarification of the phenotypic characteristics and anti-tumor activity of *Hedyotis diffusa*, Am. J. Chin. Med. 39 (2011) 201–213, https://doi.org/10.1142/S0192415X11008750.
[10] M. Ganbold, J. Barker, R. Ma, L. Jones, M. Carew, Cytotoxicity and bioavailability studies on a decoction of *Oldenlandia diffusa* and its fractions separated by HPLC, J. Ethnopharm. 131 (2010) 396–403, https://doi.org/10.1016/j.jep.2010.07.014.
[11] S.K. Vareed, R.E. Schutzki, M.G. Nair, Lipid peroxidation, cyclooxygenase enzyme and tumor cell proliferation inhibitory compounds in *Cornus kousa* fruits, Phytomedicine 14 (2007) 706–709, https://doi.org/10.1016/j.phymed.2006.09.006.
[12] M.K. Shanmugam, X. Dai, A.P. Kumar, B.K.H. Tan, G. Sethi, A. Bishayee, Ursolic acid in cancer prevention and treatment: Molecular targets, pharmacokinetics and clinical studies, Biochem. Pharm. 85 (2013) 1579–1587, https://doi.org/10.1016/j.bcp.2013.03.006.
[13] S.H. Safe, P.L. Prather, L.K. Brents, G. Chadalapaka, I. Jutooru, Unifying mechanisms of action of the anticancer activities of triterpenoids and synthetic analogs, Anti-cancer Agents Med. Chem. 12 (2012) 1211–1220, https://doi.org/10.2174/187152012803833099.
[14] J.A.R. Salvador, A.S. Leal, A.S. Valdeira, B.M.F. Goncalves, D.P.S. Alho, S.A.C. Figueiredo, S.M. Silvestre, V.I.S. Mendes, Oleanane-, ursane-, and quinone methide friedelane-type triterpenoid derivatives: recent advances in cancer treatment, Eur. J. Med. Chem. 142 (2017) 95–130, https://doi.org/10.1016/j.ejmech.2017.07.013.
[15] Q. Huang, H. Chen, Y. Ren, Z. Wang, P. Zeng, X. Li, J. Wang, X. Zheng, Anti-hepatocellular carcinoma activity and mechanism of chemopreventive compounds: ursolic acid derivatives, Pharm. Biol. 54 (2016) 3189–3196, https://doi.org/10.1080/13880209.2016.1214742.
[16] M. Labbozzetta, R. Baruchello, P. Marchetti, M.C. Gueli, P. Poma, M. Notarbartolo, D. Simoni, N. D'Alessandro, Lack of nucleophilic addition in the isoxazole and pyrazole diketone modified analogs of curcumin; implications for their antitumor and chemosensitizing activities, Chem. Biol. Interact. 181 (2009) 29–36, https://doi.org/10.1016/j.cbi.2009.06.005.
[17] P. Poma, M. Notarbartolo, M. Labbozzetta, R. Sanguedolce, A. Alaimo, V. Carina, A. Maurici, A. Cusimano, M. Cervello, N. D'Alessandro, Antitumor effects of the novel NF-kappaB inhibitor dehydroxymethyl-epoxyquinomicin on human hepatic cancer cells: analysis of synergy with cisplatin and of possible correlation with inhibition of pro-survival genes and IL-6 production, Int. J. Oncol. 28 (2006) 923–930, https://doi.org/10.3892/ijo.28.4.923.
[18] G. Kuttan, P. Pratheeshkumar, K.A. Manu, R. Kuttan, Inhibition of tumor progression by naturally occurring terpenoids, Pharm. Biol. 49 (2011) 995–1007, https://doi.org/10.3109/13880209.2011.559476.
[19] M.K. Shanmugam, X. Dai, A.P. Kumar, B.K. Tan, G. Sethi, A. Bishayee, Oleanolic acid and its synthetic derivatives for the prevention and therapy of cancer: preclinical and clinical evidence, Cancer Lett. 346 (2014) 206–216, https://doi.org/10.1016/j.canlet.2014.01.016.
[20] D. Kashyap, A. Sharma, H.S. Tuli, S. Punia, A.K. Sharma, Ursolic acid and oleanolic acid: pentacyclic terpenoids with promising anti-inflammatory activities, Recent Pat. Inflamm. Allergy Drug Discov. 10 (2016) 21–33, https://doi.org/10.2174/1872213X10666160711143904.
[21] L. Kwong-Chiu, C. Yao-Li, L. Ping-Yi, C. Wan-Ling, Ursolic acid-induced apoptosis via regulation of the PI3K/Akt and MAPK signaling pathways in Huh-7 Cells, Molecules 23 (2018), https://doi.org/10.3390/molecules23082016.
[22] E. Onoja, I.G. Ndukwe, Isolation of oleanolic acid from chloroform extract of Borreria stachydea (DC) Hutch. and Dalziel, J. Nat. Prod. Plant Resour. 3 (2013) 57–60.
[23] G.M. Woldemichael, G. Wächter, M.P. Singh, W.M. Maiese, B.N. Timmermann, Antibacterial diterpenes from *Calceolaria pinifolia*, J. Nat. Prod. 66 (2003) 242–246, https://doi.org/10.1021/np020380x.
[24] A. Paszel-Jaworska, B. Rubi, B. Bednarczyk-Cwynar, L. Zaprutko, M. Rybczy, Proapoptotic activity and ABCC1-related multidrug resistance reduction ability of semisynthetic oleanolic acid derivatives DIOXOL and HIMOXOL in human acute promyelocytic leukemia cells, Chem. Biol. Interact. 242 (2015) 1–12, https://doi.org/10.1016/j.cbi.2015.07.011.
[25] S. Wu, T. Zhang, J. Du, Ursolic acid sentitizes cisplatin-resistant HepG2/DDP cells to cisplatin via inhibiting Nrf2/ARE pathway, Drug Des. Devel. Ther. 10 (2016) 3471–3481, https://doi.org/10.2147/DDDT.S110505.
[26] M.H. Shyu, T.Z. Kao, G.C. Yen, Oleanolic acid and ursolic acid induce apoptosis in HuH7 human hepatocellular carcinoma cells through a mitochondrial-dependent pathway and downregulation of XIAP, J. Agric. Food Chem. 58 (2010) 6110–6118, https://doi.org/10.1021/jf100574j.
[27] I.L. Acebey-Castellon, L. Voutquenne-Nazabadioko, H.D.T. Mai, N. Roseau, N. Bouthagane, D. Muhammad, E. Le Magrex Debar, S.C. Gangloff, M. Litaudon, T. Sevenet, N. Van Hung, C. Lavaud, Triterpenoid saponins from *Symplocos lancifolia*, J. Nat. Prod. 74 (2011) 163–168, https://doi.org/10.1021/np100502y.
[28] Z. Yi-Nan, Z. Wei, H. Di, S. Lei, S. Qiang, L. Jing-Ya, L. Jia, H. Li-Hong, Oleanolic acid and its derivatives: new inhibitor of protein tyrosine phosphatase 1B with cellular activities, Biorg. Med. Chem. 16 (2008) 8697–9705, https://doi.org/10.1016/j.bmc.2008.07.080.
[29] A. Niesen, A. Barthel, R. Kluge, A. Köwitzsch, D. Ströhl, S. Schwarz, R. Csuk, Antitumoractive endoperoxides from triterpenes, Arch. Pharm. Chem. Life Sci. 342 (2009) 569–576, https://doi.org/10.1002/ardp.200900051.
[30] M. Miyazawa, Y. Okuno, K. Imanishi, Suppression of the SOS-inducing activity of mutagenic heterocyclic amine, Trp-P-1, by triterpenoid from Uncaria sinensis in the salmonella typhimurium TA1535/pSK1002 umu test, J. Agric. Food Chem. 53 (2005) 2312–2315, https://doi.org/10.1021/jf035430y.
[31] S. Seo, Y. Tomita, K. Tori, Carbon-13 NMR spectra of urs-12-enes and application to structural assignments of components of Isodon japonicus tissue cultures, Tetrahedron Lett. 1 (1975) 7–10, https://doi.org/10.1016/S0040-4039(00)71763-1.
[32] C.M. Ma, S.Q. Cai, J.R. Cui, R.Q. Wang, P.F. Tu, M. Hattori, M. Daneshtalab, The

cytotoxic activity of ursolic acid derivatives, Eur. J. Med. Chem. 40 (2005) 582–589, https://doi.org/10.1016/j.ejmech.2005.01.001.
[33] S.C.B. Gnoatto, L. Dalla Vechia, C.L. Lencina, A. Dassonville-Klimpt, S. Da Nascimento, D. Mossalayi, J. Guillon, G. Gosmann, P. Sonnet, (2008) Synthesis and preliminary evaluation of new ursolic and oleanolic acids derivatives as antileishmanial agents, J. Enzym. Inhib. Med. Chem. 23 (2008) 604–610, https://doi.org/10.1080/14756360802204870.
[34] S.C.B. Gnoatto, A. Dassonville-Klimpt, S. Da Nascimento, P. Galéra, K. Boumediene, G. Gosmann, P. Sonnet, S. Moslemi, Evaluation of ursolic acid isolated from Ilex paraguariensis and derivatives on aromatase inhibition, Eur. J. Med. Chem. 43 (2008) 1865–1877, https://doi.org/10.1016/j.ejmech.2007.11.021.
[35] X. Wen, H. Sun, J. Liu, K. Cheng, P. Zhang, L. Zhang, J. Hao, L. Zhang, P. Ni, S.E. Zographos, D.D. Leonidas, K.M. Alexacou, T. Gimisis, J.M. Hayes, N.G. Oikonomakos, Naturally occurring pentacyclic triterpenes as inhibitors of glycogen phosphorylase: synthesis, structure-activity relationships, and X-ray crystallographic studies, J. Med. Chem. 51 (2008) 3540–3554, https://doi.org/10.1021/jm8000949.